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Infection of the Human Monocytic Cell Line Mono Mac6 with Human Immunodeficiency Virus Types 1 and 2 Results in Long-Term Production of Virus Variants with Increased Cytopathogenicity for CD4⁺ T Cells

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The recently established human monocytic cell line Mono Mac6 expressing distinct characteristics of mature monocytes/macrophages was tested for its susceptibility to infection with human immunodeficiency virus. Inoculation of the cells with the T-cell-tropic human immunodeficiency virus strains human T-lymphotropic virus type IIIB and lymphadenopathy-associated virus type 2 led to a noncytopathic productive infection becoming apparent only after a latency period of up to 56 days. The infectibility of the Mono Mac6 cells was dependent on low levels of CD4 expression, as demonstrated by blocking experiments with various CD4-specific antibodies. Increasing with time after infection (>200 days), the cultured Mono Mac6 cells released virus variants which showed shortened latency periods when passaged onto uninfected Mono Mac6 cells. Also, cytopathogenicity for several CD4⁺ T cells of the Mono Mac6-derived virus was drastically increased; thus, the infection of the H9 cell line with low doses of virus (<0.1 50% tissue culture infective dose per cell) led to giant syncytium formation within 1 day and subsequent death of all fused cells. We propose Mono Mac6 cells as a new model for the study of human immunodeficiency virus infecting the monocyte/macrophage lineage, particularly with regard to virus-host cell interaction and the influence of cell differentiation and activation on latency and development of virulence. The human immunodeficiency virus-infected Mono Mac6 cell may also serve as a valuable tool for in vitro testing of antiviral therapies.

The human immunodeficiency virus (HIV) infects human T cells as well as non-T cells of the monocyte/macrophage lineage, B cells, follicular dendritic cells, and microglia, and there is evidence that bone marrow stem cells and even fibroblasts may be infected with HIV (17).

In vitro HIV infection of T cells expressing surface CD4 molecules causes cell fusion and syncytium formation and results in death of the fused cells (18). In monocytes and macrophages which express only a few CD4 molecules on their surfaces, HIV infection induces no detectable cytopathic effects (9). HIV can persist for prolonged periods in monocytes and macrophages (12, 19). It has been suggested that these cells may thus serve as a major HIV reservoir in vivo. Furthermore, when circulating in the blood, they may play an important role in dissemination of the HIV infection throughout the organism, particularly to the central nervous system (13, 16, 24, 26).

To study mechanisms of HIV infection in monocytes/macrophages and to investigate their role in the maintenance of virus latency, as well as the influence of host cell differentiation on HIV pathogenesis, thus far only primary cultures of monocytes/macrophages or dendritic cells from peripheral blood or cell lines representing the early monocytic stages of differentiation such as U937 (32) or THP-1 (34) have been used (1, 3, 7, 13, 16, 24).

Thus, the recently established mature human monocytic cell line Mono Mac6, derived from a patient with monocytic leukemia, may constitute an informative cell model for

the study of HIV-host cell interaction. This line shows characteristics of mature blood monocytes (phagocytosis, Fc receptor, and CD14 marker) and expresses minimal amounts of CD4 at the cell surface (36).

Mono Mac6 cells growing in suspension culture were maintained in RPMI 1640 medium supplemented with 20% fetal calf serum, nonessential amino acids, 1 μ M oxalacetate, and 1 μ M pyruvate (complete medium) at 37°C in 5% CO₂ as described before (36). Every 3 to 4 days cells were split 1:3 and fresh medium was added. The human T-lymphoblastoid cell lines H9 and K37 and the HIV type 1 (HIV-1) strain human T-lymphotropic virus type IIIB (HTLV-IIIB) (8) were originally obtained through the courtesy of R. C. Gallo and M. Popovic, National Institutes of Health, Bethesda, Md., and HIV-2 strain lymphadenopathy-associated virus type 2 (LAV-2) (4) propagated in CEM cells was kindly provided by L. Montagnier, Institute Pasteur, Paris, France, and was passaged in H9 cells. Cell lines H9, Molt4, and MT4 (15) were maintained in RPMI 1640 medium with 10% fetal calf serum at 37°C in 5% CO₂. All cell lines, when contaminated with mycoplasma during handling, were treated with fresh human serum, and absence of mycoplasma infection in each HIV-infected and control culture was controlled every 2 weeks by using Vero cells as an indicator culture system (35) and occasionally electron microscopy of HIV-infected Mono Mac6 cells.

Tissue culture supernatants of recently infected HTLV-IIIB- and LAV-2-infected H9 cells were harvested 7 to 8 days after infection, clarified by centrifugation (750 \times g for 10 min), and stored as virus stock at -70°C. The tissue

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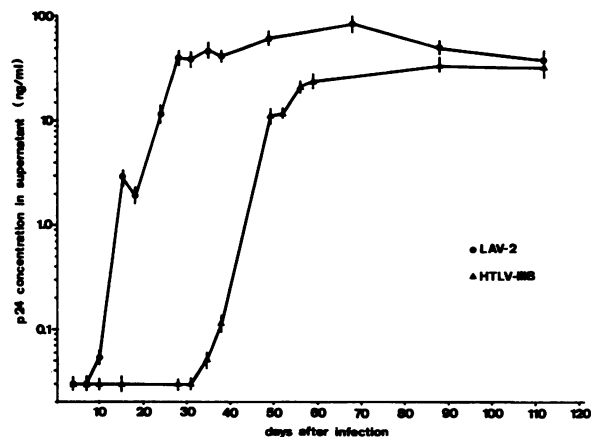


FIG. 1. Kinetics of onset and long-term production of HIV antigen p24 by HTLV-IIIB (▲)- and LAV-2 (●)-infected Mono Mac6 cells.

culture infectious dose 50% endpoint (TCID₅₀) was determined in microdilution plates by inoculating quadruplicate wells containing 5×10^4 HTLV-I genome-containing MT-4 cells (15) per well with undiluted and serial 10-fold dilutions of cell-free supernatants. MT-4 cell cultures were monitored daily microscopically for HIV-induced cell fusion and syncytium formation. The TCID₅₀ was defined as the last dilution which induced syncytium formation at day 6. Viral p24 antigen was determined in cell-free supernatants by the Du Pont HIV p24-enzyme-linked immunosorbent assay system (New England Nuclear, Dreieich, Federal Republic of Germany) detecting antigens of HIV-1 as well as HIV-2.

To establish HIV-1 and HIV-2 infections in Mono Mac6 cells, 2×10^5 cells in 1 ml of complete RPMI 1640 medium were incubated at 37°C for 30 min with a 1-ml supernatant of HTLV-IIIB- or LAV-2-infected H9 cells (0.1 to 1.0 TCID₅₀ per Mono Mac6 cell). Unbound virus was removed by washing and centrifugation of the cells at $400 \times g$ three times. The cell pellet was suspended in 2 ml of complete medium and plated into a well of 24-well plastic plates for further incubation at 37°C in 5% CO₂. Every 3 to 4 days 1 ml of the suspended cell suspension was removed and fresh medium was added. Harvested samples of cell-free supernatants were titrated for p24 antigen content and TCID₅₀ as described above.

Depending on the initial infectious dose, virus release into the culture supernatant was detected 7 to 21 days after infection with LAV-2, but not before days 21 to 56 after infection with HTLV-IIIB. After its first detection, virus production increased steadily over a period of 10 to 20 days, reaching levels of 30 to 100 ng of p24 per ml (Fig. 1) and infectious titers of 2×10^3 to 1×10^4 TCID₅₀ per ml of supernatant when assessed on MT-4 cells. After this phase, rather constant levels of p24 viral antigen (>30 ng/ml) and infectious titers (> 5×10^3 /ml) were observed for more than 300 days. Thus, the amount of infectious virus produced is about one-tenth of that released by infected H9 cells (see Table 2).

Infected, virus-producing Mono Mac6 cells did not exhibit major changes in growth behavior or morphology except for the occasional occurrence of up to 10% discretely enlarged cells. Less than 0.1% of the cells could be identified as multinucleated cells.

Thin-section electron microscopy (10) of Mono Mac6 cells

220 days after HIV inoculation revealed morphologically mature HIV particles either released from the Mono Mac6 cells or predominantly contained within cytoplasmic vacuoles (Fig. 2). These vacuoles were morphologically comparable to those described (12, 13) for HIV-infected peripheral blood monocytes/macrophages. Occasionally, budding from the plasma membrane to the extracellular space or into vacuoles was observed. Viral buds were densely studded with envelope knobs (Fig. 2a), while morphologically mature particles showed varying degrees of loss of envelope projections (11).

Cytoplasmic viral antigen was detected in <10 to a maximal 30% of the chronically infected Mono Mac6 cells (Fig. 3) by alkaline phosphatase-anti-alkaline phosphatase (APAAP) immunocytochemistry (5) with monoclonal antibodies specific for the p24 antigen of HIV-1 and HIV-2 (22, 23).

In view of the described low levels of CD4 expression in Mono Mac6 cells (36), it was of interest to know whether primary HIV infection is still dependent on the presence of this receptor. Using the APAAP technique, we found that 2 to 8% of the uninfected Mono Mac6 cells exhibited weak staining with monoclonal anti-CD4 antibodies MT151 and MT310 (27), which is consistent with earlier flow cytometry studies (36). The low expression of CD4 was further confirmed by Northern (RNA) blot analysis of total and poly(A)-enriched RNAs exhibiting no hybridization signal with a CD4-specific probe (data not shown).

To block the CD4 receptor, we preincubated Mono Mac6 cells (2×10^5 /ml) for 60 min at 37°C in complete medium containing 20 µg of purified anti-CD4 monoclonal antibodies (MT151, MT310, and MT406). MT151 and MT310 had been found previously to block HIV-1 or HIV-2 infection of H9 cells (30), while MT406, which recognizes a CD4 epitope not involved in viral gp120 binding (Rieber et al., unpublished data), had no effect. After preincubation, the treated Mono Mac6 cells were infected with 1.0 TCID₅₀ of HTLV-IIIB or LAV-2 passaged in H9 cells per cell and further incubated at 37°C for 30 min in the presence of monoclonal antibodies. After washing and sedimentation, cells were suspended in complete medium containing 10 µg of the appropriate monoclonal antibodies per ml. Furthermore, the cells were maintained in medium containing 10 µg of monoclonal antibodies per ml for up to 60 days.

In four independent experiments utilizing MT151 and MT310, no HIV replication was observed for up to 160 days after infection (Table 1), while HIV infection and replication were not affected in the presence of mouse immunoglobulin G isotype controls or in the presence of 20 µg of MT406 per ml. These results indicate that HIV infection of Mono Mac6 cells depends on low levels of CD4.

In contrast, preliminary experiments with subneutralizing doses of an anti-HIV-2 serum from an asymptomatic HIV-2-infected patient indicate that, in the presence of blocking concentrations of anti-CD4 monoclonal antibodies, infection of Mono Mac6 cells with antibody-coated HIV-2 may also occur via Fc receptors (data not shown).

HIV-1 and HIV-2 strains produced by long-term infected Mono Mac6 cells exhibited at least two striking differences in biological properties when compared with the original strains grown in H9 cells. First, when cell-free supernatant harvested later than 150 days after infection of HTLV-IIIB- or LAV-2-infected Mono Mac6 cells (containing 5×10^3 to 1×10^4 TCID₅₀ and 40 to 90 ng of p24 antigen per ml) was used to infect Mono Mac6 cells, a significantly shortened latency period of 4 to 6 days versus 21 to 56 days for primary

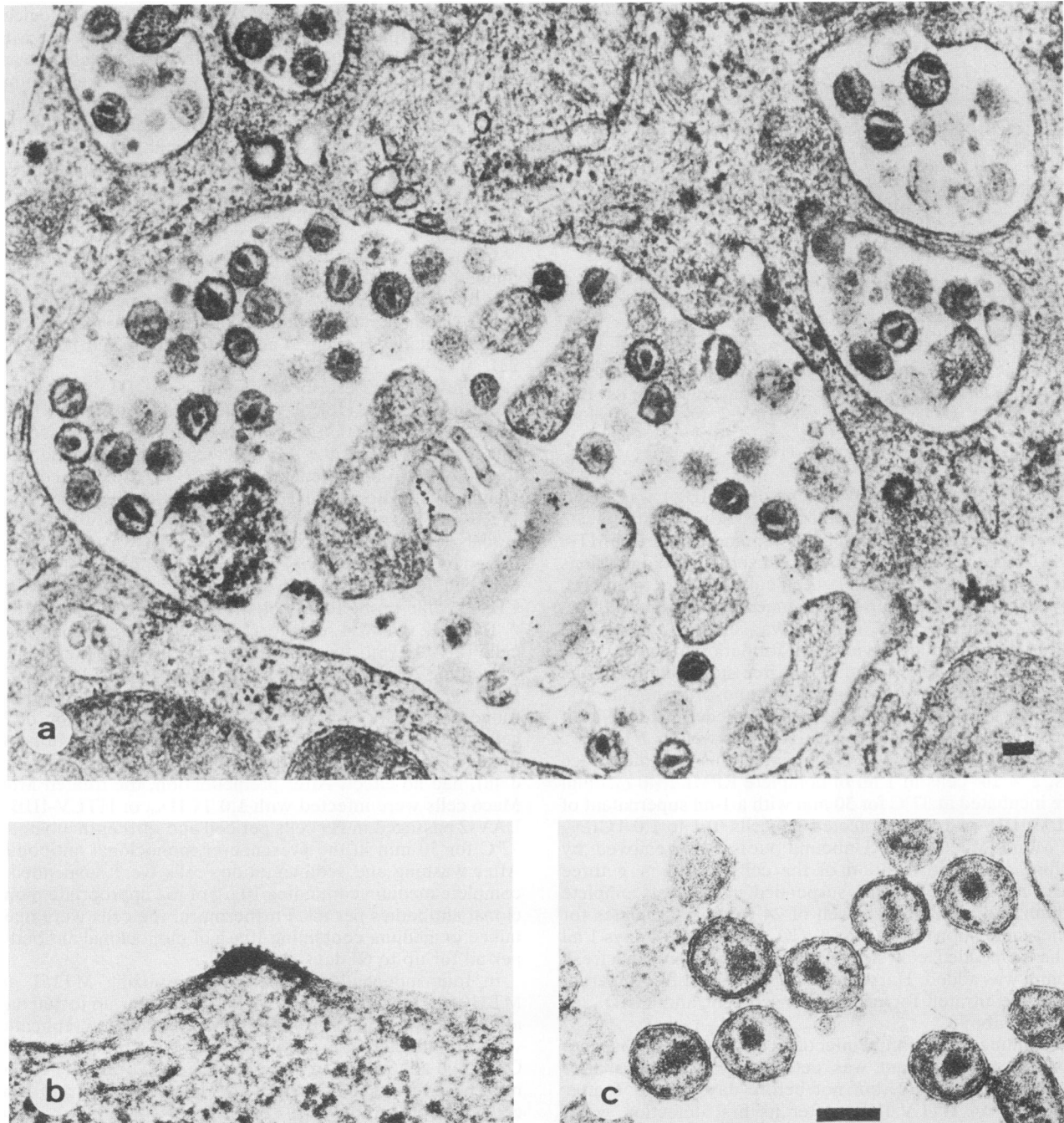


FIG. 2. Thin-section electron microscopy of Mono Mac6 cells 230 days after infection with LAV-2. (a) Large cytoplasmic vacuoles are filled with numerous mature HIV particles. (b) Occasionally, virus formation can be observed at the cell surface. The budding HIV-2 is densely studded with envelope projections, while the p55 *gag* protein precursor material is closely attached to the prospective viral envelope. (c) Morphologically mature HIV-2 particles characterized by their cone-shaped cores show a relatively high number of envelope projections. Bars, 100 nm.

infection with H9 cell-derived virus was observed. Second, in six independent inoculation infection experiments, it was found that the HIV-1 and HIV-2 strains released later than about 150 days after infection from long-term cultured, infected Mono Mac6 cells displayed a distinctly increased cytopathogenicity for CD4⁺ T-cell lines such as H9 or Molt4 and that these cells died within a few days.

To assess changes in cytopathogenicity for H9 cells in more detail, supernatants were harvested from Mono Mac6 cells later than 200 days after infection. Cell-free supernatants were titrated for p24 antigen content and TCID₅₀ on MT-4 cells as described above, and 0.1 ml of undiluted or serially 10-fold-diluted supernatant was added to replicate microdilution wells containing 2×10^4 H9 cells per well. For

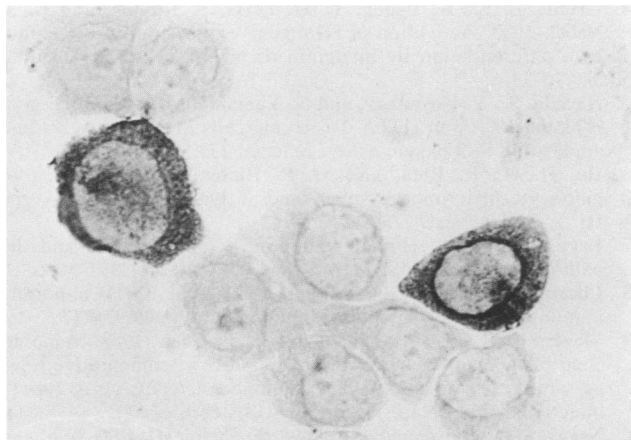


FIG. 3. Immunocytochemistry of LAV-2-infected Mono Mac6 cells (36) at day 180 after infection: APAAP technique (5), using anti-p24 monoclonal antibody 6-D-12 (23).

comparison, cell-free supernatants from H9 cells infected with HTLV-III_B and LAV-2 were used. Cultures were monitored daily for syncytium formation. Syncytium formation occurred as soon as 6 to 24 h after infection in H9 cell cultures exposed to 0.001 to 0.1 TCID₅₀ of Mono Mac6 cell-derived virus strains per cell (Table 2), whereas 0.01 to 1.0 TCID₅₀ of the H9 derived virus strains per cell induced syncytium formation only 3 to 4 days later. The Mono Mac6 cell-derived virus variants induced giant confluent syncytia which involved several hundred cells each and virtually all H9 cells in the cultures. Syncytium formation resulted 2 to 3 days later in dead giant cells containing condensed nuclei. Cell viability as assessed visually and by trypan blue exclusion at day 6 after infection was <1% in H9 cells infected with the Mono Mac6 cell-derived virus, while >80% of the H9 cells infected with up to 1,000-fold-higher infectious doses of H9 cell-derived virus were viable at that time (Table 2). Another sign for the increased and early cytotoxic effect of Mono Mac6 cell-derived HIV strains was the absence of p24 antigen production of such infected H9 cells compared with a steady increase of p24 antigen content in supernatants of H9 cells infected with HTLV-III_B or LAV-2 derived from

TABLE 1. Inhibition of HIV infection in Mono Mac6 cells by CD4-specific antibodies

Infection in presence of CD4-specific antibodies ^a	Virus replication in Mono Mac6 cells >80 days after primary infection with:			
	HTLV-III _B		LAV-2	
	p24 content (ng/ml)	TCID ₅₀ /ml	p24 content (ng/ml)	TCID ₅₀ /ml
MT151	<0.0	0 ^b	<0.1	0
MT310	<0.1	0	<0.1	0
MT406	>50	>10 ⁴	>50	>10 ⁴
None	>50	>10 ⁴	>50	>10 ⁴
Isotype control	>50	>10 ⁴	>50	>10 ⁴

^a Designation of mouse monoclonal CD4-specific and immunoglobulin G isotype control antibodies.

^b 0, No infectious virus (TCID₅₀) detectable in a 1-ml supernatant of Mono Mac6 cells.

TABLE 2. Demonstration of increased virulence for H9 cells of HIV-1 and HIV-2 strains released from Mono Mac6 cells >200 days after infection with HTLV-III_B or LAV-2

Virus strain	Grown in cell line	Infectious dose ^a		Start of syncytium formation (days)	% Viability on day +6
		ng of p24 per ml	TCID ₅₀ /cell		
HTLV-III _B	H9	520.0	1.0	3	80-95
		52.0	0.1	3	80-95
		5.2	0.01	>4	80-95
	Mono Mac6	36.0	0.1	<1	<1
		3.6	0.01	1	<1
		0.4	0.001	2	<1
LAV-2	H9	660.0	0.1	3	80-95
		66.0	0.01	3	80-95
		6.6	0.001	4	80-95
	Mono Mac6	89.0	0.1	<1	<1
		8.9	0.01	1	<1
		0.9	0.001	2	<1

^a Content of p24 antigen and TCID₅₀ were determined per milliliter of cell-free culture supernatant harvested 7 days after infection of H9 cells and later than 200 days after infection of Mono Mac6 cells and used for infection of H9 cells.

H9 cells. Thus, the phenotype as well as the kinetics of the cytopathic effect were drastically altered when the Mono Mac6 cell-passaged virus was used.

Whether the reproducible appearance of cytotoxic HIV-1 and HIV-2 strains in long-term cultured Mono Mac6 cells reflects a mutation or selection of the virus during multiplication in the CD4-deficient monocytic cell line or whether it reflects another mechanism involving host cell differentiation factors remains to be analyzed on the molecular genetic level.

It has been reported previously (3, 6, 7, 14, 25) that a productive HIV infection in monoblastoid cell line U937 is influenced by the state of cellular differentiation. According to these observations, virus production ceased 2 to 3 days after infection and was reinduced after differentiation induction. Also, in other lentivirus infections, such as caprine arthritis encephalitis virus, virus production appears to be determined by the stage of differentiation of the monocytic cells harboring the virus (20). The increased cytopathogenicity of HIV for CD4⁺ T-cell lines becomes apparent only after the initial latency period: whether this reflects the generation of more cytopathic HIV strains isolated from individual patients during late stages of their disease remains to be investigated (2, 28, 33).

The question of whether the generation of more cytotoxic HIV variants is enhanced by a particular monocytic cellular environment in defined stages of cellular differentiation can now be addressed.

To our knowledge, this is the first report on the reproducible development of HIV variants with increased cytopathogenicity for H9 cells of H9-derived HTLV-III_B and LAV-2 strains by long-term culturing in a low-CD4-expressing monocytic cell line.

From the experiments described, the Mono Mac6 cells appear to represent an informative model for virological and molecular studies on HIV pathogenetic mechanisms. Furthermore, they should be a valuable tool for preclinical testing of antiviral drugs which may act differently in HIV-

infected T cells compared with chronically infected monocytes/macrophages.

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